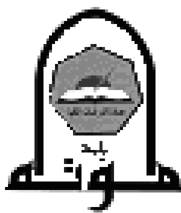


Mu‘tah University
Deanship Of Graduate Studies

Biological Characterization of active metabolites form bacteria isolated from Ain Sara-Karak

By:
Maisa’a Mahmoud Al-hourani

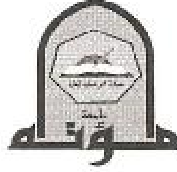
Supervised by
Prof.Dr.KhaledKhleifat

Co-advisor
Dr.Wael Al-Zereini

Thesis Submitted to the Deanship of theGraduate Studies in Partial Fulfillment of theRequirements For the Degree of Master ofScience Department of Biology

Mu’tah University, 2014

ء الآ ا ةداولداي ف الرسالة الجامعية لاتُ عبر
بالضرورة عن وجهة نظر جامعة مؤتة



MUTAH UNIVERSITY

Deanship of Graduate Studies

جامعة مؤتة

عمادة الدراسات العليا

نموذج رقم (14)

قرار إجازة رسالة جامعية

تقرر إجازة الرسالة المقدمة من الطالبة ميساء محمود الحوراني الموسومة بـــ:

Biological characterization of active metabolites form bacteria isolated from Ain Sara- karak

استكمالاً لمتطلبات الحصول على درجة الماجستير في العلوم الحياتية.

القسم: العلوم الحياتية.

	التوقيع	التاريخ	
أ.د. خالد محمد خليفات		2014/01/06	مشرفاً ورئيسا
د. وائل علي الزرعيني		2014/01/06	مشرفا مساعداً
أ.د. جمعه مطيع الشخانبه		2014/01/06	عضواً
د. عامر منور الحروب		2014/01/06	عضواً
د. خالد يوسف الشرفاء		2014/01/06	عضواً

عميد الدراسات العليا

د. علي الضمور



MUTAH-KARAK-JORDAN
Postal Code: 61710
TEL :03/2372380-99
Ext. 5328-5330
FAX:03/ 2375694
e-mail: dgs@mutah.edu.jo sedgs@mutah.edu.jo
http://www.mutah.edu.jo/gradest/derasat.htm

مؤته – الكرك – الاردن
الرمز البريدي: 61710
تلفون: 03/2372380-99
فرعي 5328-5330
فاكس 03/2 375694
البريد الالكتروني
الصفحة الالكترونية

Dedication

To my great father for giving me a lot of encouragement and inspiration.

To my wonderful mother for all the prayers and unlimited love..

To my lovely sisters: Enasand Esra'awho were such good supporters during my hard times.

To my brother: Eng. Eqbal Al-hourany.

And to my dear professors who were the beacons of my rough path in learning the new.

I am so proud of you all, and I hope that I will make you proud

Maisa'a Al-hourani

Acknowledgements

All praise is to Allah.. I’m beholden and grateful to Allah for opening the doors for me .

I would like to thank Prof Khaled M. Khliefat .

I also would like to express my gratitude to Dr.Wael Al-Zereini for his support and advice throughout this work.

I am deeply thankful for my family for providing the nice atmosphere, and support during my study.

Special thanks for AyatTarawneh for helping me .

I would like to extend my thank to my colleagues : Sakha’aDardasawi, HashemSarayreh , BaraaFrejat , WafaaShamaileh , Mr.Yaseen Al-Qaisi, TahaniHijazeen .

Finally I also thank all the stuff members of Biology Department .

Maisa’a Al-hourani

Table of Contents

Chapter Four: Results and Discussion

List of tabels

List of figures

List of abbreviations

for. Forward
H2O dist Distilled water
OD Optical density
rev. Reverse
rpm Round per minute
SDS Sodium dodecylsulfate
TLC Thin layer chromatography
UV. Ultraviolet
Vis. Visible
ATCCAmerican Type Culture Collection .

Abatract

Biological Characterization of active metabolites form bacteria isolated from Ain Sara-Karak

Maisa'aMhmoud Al-hourani

Mu'tahUniversity , 2014

Thirty three soil bacterial isolates were obtained from Al-Karak – Ainesarah. The crude extract of nine strains that showed antibacterial activity were screened for their antibacterial activity against six types of pathogenic microorganisms (*Bacillussubtilis, Escherichia coli, Staphylococcus aureus, Micrococcus luteus, Enterobacteraerugenes, Psedomonusaeroiginosa)*. One of these isolated (D12) was shown to be the most effective strain. Bacterial isolate named D12 was identified as *Arcanobacteriumpyogenes*by using RAPID CB plus kit. Optimization of the conditions for production of bioactive extract was performed. The crude extract of *A. pyogenes* D12 has slight antibacterial activity against *Micrococcus luteus* whereas other gram positive and negative ones showed resistancy toward this extracts. The crude extract of this bacterial isolate was subjected to partial purification by Thin layer chromatography.The bioactivity of the partially purified compounds was tested by agar diffusion assay and showed antimicrobial activity generally for all fractions but in similar manar to that of the crude .

الملخص

استخلاص المواد الحيوية من البكتيريا المعزولة من التربة في منطقة الكرك–عين سارة ودراسة تأثيرها كمضادات بكتيرية

ميساء الحوراني

جامعة مؤتة، 2014

تم عزل ثلاثة وثلاثين عينة بكتيرية من التربة في منطقة عين سارة في الكرك، تسعة أنواع من البكتيريا المعزولة أعطت مستخلصاتها تأثير ضد ست انواع من البكتيريا المرضية وكان مستخلص البكتيريا المعزولة التي تحمل الرمز D12 ذو نتيجة ملحوظة.
وبعد تحديد نوع البكتيريا وجدانها من نوع *(ArcanobacteriumPyogenes)* باستخدام RAPID CB plus kit .

وكان للعزلة البكتيرية التي تحمل الرمز (D12) نشاط ملحوظ .

Chapter One
Theoretical Background

1. Introduction

1.1. Natural products

Natural products are substances synthesized by living organisms such as animals, plants and microorganisms. These compounds may be derived from primary or rather secondary metabolism of these organisms (Al-Tarawneh, 2011). Due to their chemical diversity and various activities against diseases, they have been playing an important role in pharmaceutical and agricultural research (Al-Saady, 2011). Among bioactive natural products are the antibiotics. They are defined as a "secondary metabolite, produced by microorganisms, which has the ability to inhibit the growth and even to destroy bacteria and other microorganisms, in a very low concentration" (Al-Zereini, 2006).

Since the discovery of penicillin (penicillin G) in 1928 (Fleming, 1929), intensive studies, mainly on soil derived bacteria and fungi, have shown that microorganisms are a rich source of structurally unique bioactive substances (Fenical, 1993). Microbial secondary metabolites are often having unusual structures and their synthesis is regulated by nutrients, growth rate, enzyme inactivation and induction (Demain, 1998). These metabolites are produced during starvation for nutrient sources. In fact, they have played a key role in the discovery and development of many antibiotics (Motta *et al*., 2004).

Despite the large number and diversity of these compounds and due to the emergence of new infectious diseases and resistant pathogens that represent a serious problem for human life (Needham,. 1994; Cragg*et al.*, 1997; El-Banna*et al*., 2007), the need for new therapeutic compounds from nature is still urgent. In addition, according to the World health organization, over-prescription and the improper use of antibiotics has led to the generation of antibiotic resistance in many bacterial pathogens.

1.2. Aims of study

This work was initiated to achieve the following objectives:

1. Isolation of bacterial strains able to produce antimicrobial natural products.
2. Evaluating the bioactivity of their crude extracts in agar diffusion and serial dilution assays.
3. Identification the bacterial isolate of interest.

Chapter two
Literature review

2.1. Microbial secondary metabolites:

The observation of the effects of microbial secondary metabolites on pathogenic fungi and bacteria spawned the antibiotic era (Demain, 1998; Cragg*et al.,* 2001). Nevertheless, the difficulties in identifying novel structures and the problems in finding new mechanisms of action derive the golden era of antibiotics to meet its own demise. Accordingly, research into the complete biological activity profiles of antibiotics began with the intent of identifying the utility of these compounds for various pharmacological or agrochemical applications. This shift in focus expanded the search for new natural products from microbes, where microbial metabolites might be used to treat diseases other than those caused by bacteria and fungi. Microorganisms have proven to be an excellent source of novel natural products including polyketide and peptide antibiotics as well as classes of other biologically active compounds (O'Keefe, 2001).

Today, microbial metabolites are used as antineoplastic agents (e.g., mitomycin), immunosuppressive agents (e.g., rapamycin), hypocholesterolemic agents (e.g., pravastatin), enzyme inhibitors (e.g.,desferal), antimigraine agents (e.g., ergot alkaloids), herbicides (e.g., bialaphos), antiparasitic agents (e.g., salinomycin), bioinsecticides (e.g., tetranactin), and ruminant growth promoters (e.g., monensin) (Demain, 1998).

Although soil microorganisms represent the source of vast number of known antibiotics, increasing evidence has shown that the number of species cultivated from soil represents less than 1% of the total population. Therefore, uncultured species may provide a large untapped pool from which novel natural products can be discovered (Al-Saady, 2011).

2.2. Classification of antibiotics

Antibiotics can be divided into broad-spectrum and narrow-spectrum antibiotics. Tetracycline, a broad spectrum antibiotic, is active against gram positive bacteria, gram negative bacteria and even against mycobacteria (Fleming, 1980). Penicillin, on the other hand, has a relatively narrow spectrum. It can be used mainly against gram positive bacteria. Other antibiotics, such as Pyrazinamide, have an even narrower spectrum, and can be used merely against *Mycobacteriumtuberculosis.*

The mode of action of antibiotics against bacteria is performed by inhibiting certain vital processes of bacterial cells or metabolism. Thus, classification of antibiotics is divided into five major classes: 1. Cell wall

inhibitors, such as Penicillin and Vancomycin; 2. Inhibitors of genome replication, such as Fluoroquinolones, which inhibits DNA synthesis, and Rifampin, which inhibits RNA synthesis; 3.Translation inhibitors, such as Aminoglycoside; 4. Anti-metabolites, such as the sulfa drugs; 5.Antibiotics that can damage the membrane of the cell, such as Polymyxin B, Gramicidin and Daptomycin (Ying, 2007) .

2.3. Resistance to antibiotics

The availability of antibiotics has allowed the successful treatment for many bacterial infections as well as the ability to perform invasive medical procedures including surgery and chemotherapy. However, many bacterial strains have developed ways to become resistant to the currently available antibiotics (Hancock *el at.*, 2001). Many of *Staphylococcusaureus* strains are resistant to penicillin antibiotic (Breithaupt ,1999).

The misuse of antibiotics in health centers and hospitals is one of the reasons to the development of bacterial resistance to antibiotic. Moreover, pervasive use of antibiotics in animal feeds to prevent infection and stimulate growth cause emergence of resistance to traditional used drugs (Davies, 1994).

Spontaneous mutation of the targets of anti-bacterial drugs as well as the exchange of plasmids encoding antibiotic resistant genes also contributed to the resistance against antibiotic. There are many mechanisms by which bacteria avoid antibiotic treatment (Breithaupt, 1999). Genetic material can be transferred among bacteria by several means. The most common mechanisms of genetic transfer are conjugation, transformation and transduction (Alanis, 2005). These mechanisms have an important role in changing the genetic material of bacteria to increase their ability to develop their resistance to antibiotics. Plasmids provide functions that might not be encoded by bacteria. They carry genes that code for antibiotic resistance as well as genes that can enable the transfer of genes from one bacterial cell to another (Sedgley&Clewell, 2004)**.**

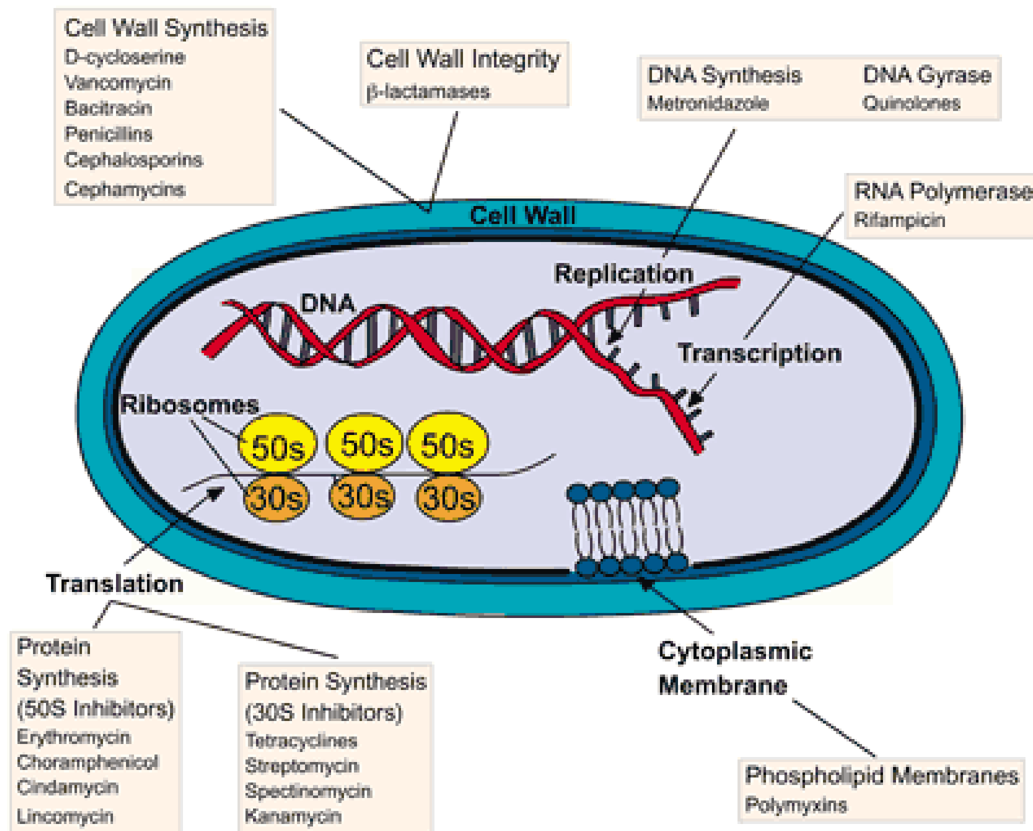


Figure 1.
Sites of action of different antibiotics
(www.wiley.com/college/pratt/0471393878/student/activities/bacterial_drug_resistance/index.html)

2.4. Mechanisms of resistance:

Bacterial antibiotic resistance is due to three reasons: (i) Modification of the active site to the target cell, (ii) Direct destruction or modification of the antibiotic by enzymes produced by the organism, and (iii) efflux of antibiotic from the cell (iv) decreased penetration (Sheldon, 2005).This is shown in figure 1.

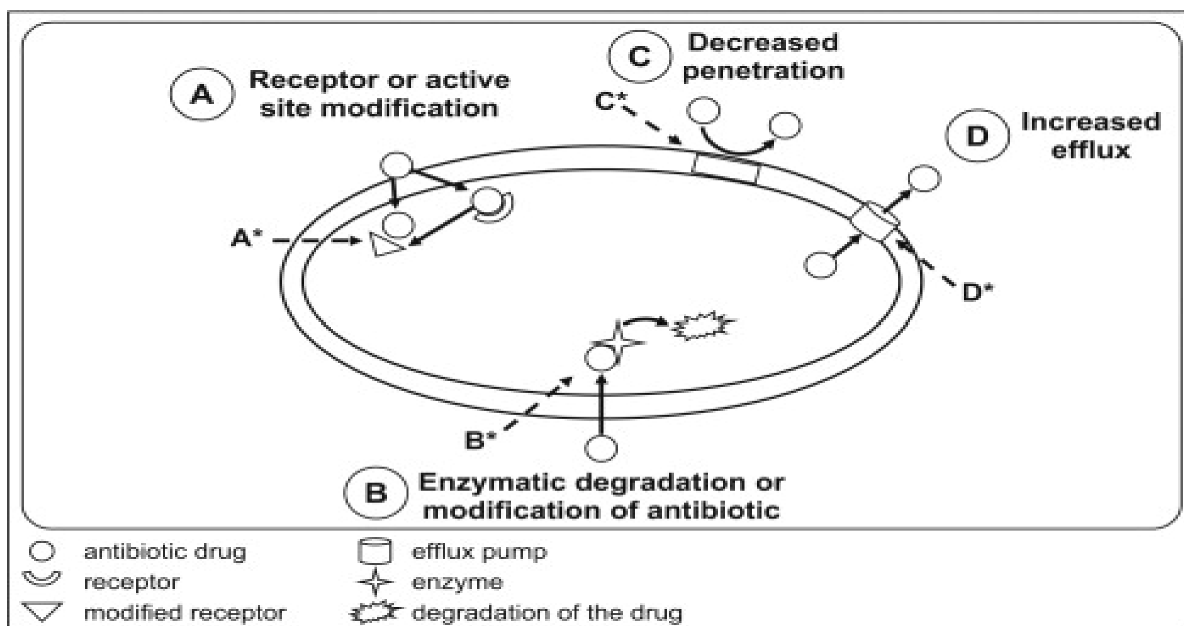


Figure2.

Mechanisms of antibiotic resistance. (Hemaiswarya, *et al*.,2008**).**

2.5. Soil bacteria as a source for natural products

Environmental conditions such as nutrient availability, soil texture, and type of vegetation cover affect the number and species of microbes in soil (Atlas and Bartha , 1998). It was reported that microorganisms such as soil bacteria and fungi are good source of natural antibiotic (Fenical , 1993 ; Dancer , 2004). Numerous antibiotics have been isolated from a variety of microorganisms. However, there still interest to identify novel antibiotics effective against pathogenic microorganisms (Islam et.al , 2009). Several screening programs have been developed for discovering of new species or unknown bioactive substances. One of these approaches is isolation and screening of microorganisms from relatively unknown or unstudied areas (Ensign & Emeritus, 2002; Dehnad et.al , 2010).

Filamentous soil bacteria belonging to the Actinomycetes are widely recognized as industrially important microorganisms because of their ability to produce many kinds of novel secondary metabolites including antibiotics (Ceylan *et al*., 2008). Each strain of Actinomycetes has the genetic potential for the production of 10 to 20 secondary metabolites (Sosio*et al*., 2000; Bentley *et al*., 2002). It was estimated that70% of all known drugs have been isolated from this species (Miyadoh, 1993).

Actinomycetes population has been identified as one of the major group of soil population which may vary with soil type. *Streptomycetes* are the most well known genus of Actinomycetes family which always has been

notified because of their ability to produce and secrete a large variety of industrial, medical, biotechnological and agricultural secondary metabolites. They produce over two thirds of the clinically useful antibiotics of natural origin (e.g. Neomycin and Chloroamphenicol) (Dehnad et.al, 2010).

The bioactive compounds isolated from actinomycetes are classified into several major structural classes such as amino glycosides (e.g. streptomycin and kanamycin), ansamycins (e.g. rifampin), anthracyclines (e.g. doxorubicin), β- lactam (e.g. cephalosporins), macrolides (e.g. erythromycin), and tertracycline (Najwade *et al*., 2010).

*Pseudomonas fluorescens–pseudomonasputida*bacteria obtained from soil produce metabolites chelating the environmental iron, siderophores, and thus making it unavailable to pathogens (Battu and Reddy , 2009).

2.6 Microbial bioactive metabolites from Jordanian soil

Different soil habitats in the Hashemite kingdom of Jordan spawned bacterial strains with various bioactivities.El-Banna (2007) isolated two soil *Corynebacterium* species, *C.kutscheri*and*C. xerosis*, from Jerash. They were found to produce several antimicrobial substancesagainst fungi and bacteria such as *Fusariumoxysporium*, *Candidaalbicans* and *Escherichia coli*. He and his colleagues showed that substances produced by bacteria isolated from different Jordanian sources have antibacterial activity against methicillin-resistant *S. aureus*(El-Banna*et al.*, 2007). In addition, soil streptomycetes were able to inhibit the growth of multi-drug resistant *Pseudomonas aeruginosa*(Saadoun*et al.*, 2008). Moreover, Actinomycin C2 and actinomycin C3 were reported to inhibit the growth of both *M. luteus*and *S. aureus*(Falkinham*et al.*, 2009).

In our laboratory,*Brevibacterium*sp. 2A2, isolated from Al-Tafiela region, showed antibacterial activity against gram positive bacteria *B. subtilis*, *M. luteus* and *S. aureus* and *proteus vulgaris* (Al-Saady, 2011). *Bacillus* sp. 1A1, isolated from soil of Al-Karak region, was highly active against *P. Vulgaris*, *Micrococcus luteus* and *S. aureus*(Al-Tarawneh, 2011).

Chapter Three
Materials and Methods

3.1 Materials :

3.1.1 List of chemicals :

All chemical substances that were used in this work and their companies are listed in table (3.1)

Table (3.1) :
all chemical substances that were used in this work

Chemicals	Company
Ethanol	UNI-CHEM Chemical Reagents
Methanol	ScharlauChemie S.A
Ethyl acetate	Carbon Group
Nutrient broth	ScharlauChemie S.A
Agar	HIMEDIA , India
Tryptone	Fluka Chemie AG CH -9470 Buchs
Yeast extract	Fluka Chemie AG CH -9470 Buchs
Sodium Sulfate	Lonover House , England
Hydrochloric acid	TEDIA Company , INC
Iodine	Fluka Chemie AG CH -9470 Buchs
Safranin T	Ridel-de Haën , Germany
Crystal violet	Janssen Chimica
Agrose	Bio Basic INC
Starch	Gainland Chemical Company , U.K
NaCl	C.B.H Lab Chemicals
NaOH	Gainland Chemical Company , U.K
Hydrogen Peroxide 30%	Janssen Chimica
Acetone	Gainland Chemical Company , U.K
Toluene	Gainland Chemical Company , U.K

3.1.2 Culture Media and Solutions :

3.1.2.1 Culture Media used for cultivation of bacteria

Several culture media were used for culturing the bacteria during this study. The pH of media was controlled using 1N NaOH and 1N HCl , before autoclaving. The amounts were dissolved in 1L of distilled water:

1- Nutrient broth medium (NB)

A - Nutrient broth	13 g
B - pH	7.6 ± 0.2

2- Nutrient agar medium (NA)

A - Nutrient broth	13 g
B - Agar	16 g
C - pH	7.6 ± 0.2

3- Luria-Bertani medium (LB)

A - Yeast extract	5 g
B - Tryptone5 g	
C - NaCl10 g	
D - pH	7.2 ± 0.2

3.1.2.2 Solutions :

1- Normal saline solution

a-NaCl9 g

b - Distilled H_2O 1000 ml

Gram Staining was used and consisted of three Solution :

1- Crystal violet solution

Solution A :

a- Crystal violet	2 g
b- Ethanol (95%)	20 ml

Solution B :

a- Ammonium oxalate	0.8 g
b- H_2O dist	80 ml

= Solutions A & B mixed torether

2- Iodine solution

a-KI 2 g

b-$I_2$1 g

c-H_2O dist 300 ml

3- Safranin solution

a- Safranin1 g

b-H_2O dist 100 ml

3.1.3 Testing microorganisms used in the bioactivity test:
Six stains of bacteria were used to determine the antimicrobial activity of the crude extracts obtained from the bacterial strain that were isolated from soil, (Table 3.2)

Table(3.2)
Bacterial isolates used in the bioactivity test

Organisms	Strain NO.	Temperature (◦C)
Gram positive		
Micrococcus Luteus	ATCC 10240	27
Staphylococcus aureus	ATCC 43300	37
Bacillus subtilis	ATCC 6633	37
Gram negative		
Enterobacteraerugenes	ATCC 13048	27
Psedomonusaerugenes	ATCC	
E-coli	ATCC 13048	37

3.2 Methods :
3.2.1 Collection of soil sample :
Soil samples were collected from Al-Karak waste water treatment station (AlKarak – Ainesarah / Jordan). The samples were collected from different depths in each site (5 cm, 25 cm, and 40 cm) and placed in sterile bottles. These bottles were opened directly under the surface of soil to prevent contamination. The samples were transported to laboratory in ice box.

3.2.2 Isolation of microorganism from soil samples :
1 g of soil was added to the 10 ml of distilled waster and shaked it to make the soil bacteria more suspended. Then a series of dilution in 9mL of the sterilized distilled saline-containing test tubes were made. After then 100 µl of each dilution was spreaded on sterile nutrient agar plates for bacterial isolation based on colony morphology .

3.2.3 Bacterial Culture Maintenance:

These isolated bacteria were sub-cultured every month to keep them alive. For long term preservation, the isolated bacteria were kept in agar slant medium at 4 ◦C.

3.2.4 Identification of bacterial strains :

To identify the isolated bacterial stains the following studies were perfomed :

Morphological characterization:

Morphological characterization was performed with a microscope (Nikon/Japan) for bacterial strains grown for 48-72 hours on nutrient agar plates. Several features were used for characterization such as colony form, surface characteristics, consistency and type of margins and Elevation.

Gram-staining was examined under a compound light microscope (Olmpus CX2, China) at 1000x. Gram-reaction was done according to well-known procedures (SöƁmuth*et.al*. 1999)

Biochemical and physiological characterization :

Biochemical and physiological characterization were applied using standard procedures (Baumann *et al.* , 1972 ; Bowman , 1997 ; SöƁmuth*et al*. , 1999). Production of acid from different carbohydrates was done as described by Helmke&Weyland (1984). The inoculated media were scored for changes in color (indicating acid production) every week for a duration of two months. The test for utilization of organic compounds as sole carbon source and as sole nitrogen source was done as described by Helmke&Weyland (1984) in BMS-N medium and BMS medium respectively. Hydrolysis of DNA was detected in DNase agar medium plate. Hydrolysis of starch was detected in
nutrient agar plate supplemented with 5% starch using I2-Gram stain solution.

Hydrolysis of gelatine was also detected using gelatine agar plate .

3.2.5 Cultivation of bacterial samples in small scales

Cultivation of isolated bacterial strains was performed as mentioned in Al-Zereini (2006). The flasks were incubated at 27 ◦C on a orbital shaker (Forma Orbital Shaker , Thermo electron cooperation , USA).

Briefly , 1-L Erlenmeyer flasks containing 500 ml of LB media were inoculated separately with a single colony. This step was done for each isolate. A single colony from a well grown agar plate was used an

inoculums. Immediately after the inoculation of each bacterial strain , 50 ml sample and thereafter-daily samples were taken for OD measurement. When the OD decreased, the culture was harvested by centrifugation for 15 min at 3500 rpm (Sorvall®RC-5B,Dupont company/USA). The supernatant was divided into two equal volumes , the pH of the first half was adjusted to PH 4 with 1 N HCL and the other half was adjusted to pH 8 with 1 N NaOH. The supernatants were extracted with equal volumes of ethyl acetate.

3.2.6 Determination of antimicrobial activity of crude extracts of isolates:

Antimicrobial activity of each extract was determined by using the agar diffusion assay. 300 , 500 µg of crude extract was applied onto 6 mm sterile filter paper disc .

3.2.7 Large – scale cultivation of bacterial strain :

Bacterial strain of interest was inoculated in (5 X 2 L) Erlenmeyer flasks containing 1 L of LB media depending on the screening results.

During cultivation process, 50 ml daily samples and therafter daily samples were taken in intervals of 12-18 hours and used to measure the OD_{580}nm and pH values . The cultivation was stopped directly after the OD value decrease , the culture was centrifuged for 15 min at 4000 rpm. The supernatant was extracted with an equal volume of ethyl acetate.

After separation , the organic phase was dried over sodium sulphate (anhydrous) and concentrated in rotatory evaporation (for drying out the samples). The resulting crude extract was dissolved in methanol to a final concentration of 100 mg/ml and stored at 4◦C.

3.2.8 Fermentation parameters :

Culture turbidity

Growth of the bacterial cells indicated by the degree of turbidity was evaluated by measuring the optical density of the culture at 580 nm (OD_{580}nm) . Measurements of the OD were done with 10-fold diluted samples with a spectrophotometer (UV. Spectrometer , Lambda 16, Perkin-Elmer, Langen).

pH - value measurement :

Changes in pH value were performed using pH-meter (pH 523 , WTW , Germany)

Thin Layer Chromatography (TLC) :

Thin Layer Chromatography was carried out with the crude extract on Aluminium plates impregnated with silica gel 60 (20 X 20 cm).

The following mobile phases were used to develop the spotted samples on the plate :

Toluene : acetone : acetic acid (70 : 30 : 1)

The crude extract (10 µl) was spotted 1 cm above the base of the TLC plate. The solvent front was allowed to run in the plate , then dried from the solvent. The bands were detected with the UV light at 360 nm.

3.2.9 Biological characterization :

Agar diffusion test and bioautography :

The antimicrobial activities of crude extracts were determined by agar diffusion test. The test microorganisms were cultured in a nutrient broth and incubated at the appropriate temperature for 18 hours. Bacterial test plates were prepared in nutrient agar medium in density of 10^6 bacterial cells/ml.

A defind concentration of crude extract was applied onto filter paper discs (6 mm). The discs were placed on the top of the agar plates surface which were then incubated for 24 hours at the appropriate temperature. The antimicrobial activity was determined by measuring the diameter of the inhibition zone.

In bioautography , The crude extract was applied on TLC plates. The plates were run in organic mobile phase as described 3.2.9.2. The developed bands were cut in to small pieces (0.5 cm). Each piece was placed with the silica side on the agar test plate seeded with the test organisms. The plates were incubated for 24 hours at the appropriate temperature ,the antimicrobial activity was then determined.

Photographic documentation :

The photomicrographs showing the morphology of bacterial strain were taken with the light compound microscope OPTIKA (model N400- FL,Italy) provided with camera (Canon PC 1201 , China)

Chapter Four
Results and Discussion

4.1Screening of bacterial isolates:

Throughout this study thirty three bacterial isolates were obtained from soil samples. Growth Parameters for these isolates were taken including OD and pH . The crude extract of nine bacterial strains showed antibacterial activity. Their bioactive summary against the pathogenic bacteria is shown in Table (4.1)

These results are in conformity with those of Hentschel et al. (2006) who indicated that most of bacterial isolates showed antagonistic effects against bacteria more than fungi specially the strains isolated from hospitals as*M.luteus, Staplylococcus epidermis* and *S. aureus*. The ability of bacterial filtrate to antagonize the pathogenic bacteria may be referred to that some microbial species produce antimicrobialsubstances which affect only the systematically related species (Mohammed and Sheikh, 2010).

Table (4.1)

Bioactivity of crude extract from soil bacteria isolates measured as inhibition zone (mm)

	Inhibition zone (mm)500 µg/disc				
Bacterial isolates	*E.coli*	*B.subtilis*	*M.luteus*	*S.aureus*	*E.aurogenes*
D1(PH4)	_	_	10	11	_
D1(PH8)	_	_	12	12	_
D3(PH4)	_	_	10	_	
D3(PH8)	_	_	11	11	_
D4(PH4)	_	_	14	12	_
D4(PH8)	_	_	15	15	_
D6(PH4)	_	_	9	9	_
D6(PH8)	_	_	11	10	_
D9(PH4)	_	_	10	_	_
D9(PH8)	_	_	11	_	_
D11(PH4)	_	_	_	13	_
D11(PH8)	___15	_			
D12(PH4)	_	_	8	14	_
D12(PH8)	_	_	11	_	_
D13(PH4)	_	_	_	13	_
D13(PH8)	_	_	_	13	_
D15(PH4)	_	_	_	11	_
D15(PH8)	_	_	11	_	_

It is predicted from the shown that Gram positive bacteria were the most susceptible for the bioactive crude extracts tested. The Gram negative

resistance toward crude-extract bioactive could be attributed to the reduced permeability through the outer membrane to these bacteria, thus our results was in agreement with that obtained by my ex lab mate Alsaady, (2011) and Dehnad*et al.,* (2010). Therefore Bacterial isolate D12 showed the best activity against *M.luteus*based on inhibition zone shown by this extract. Therefore, it was selected for further studies as shown in Figure (1)



Figure 3

:**Antimicrobial activity of A*rcan. Pyogenes*sp. D12 in agar diffusion test. against M.luteus**

4.2 Identification of Isolate D12 as *Arcan.pyogenes*sp.

The bacterial isolates were identified according to Bergeys manual (Holt et al., 1994). D12 was isolated from the soil at 15 cm depth. Strain D12 grow as small, white and circular colonies with entire margin with umbonate elevation and smooth shiny surface on nutrient agar media . The cells are Gram positive. They are 0.8-2.5 µm long and 0.6-1.0 µm wide . It is oxidase positive and catalase positive, non-spore forming . It was identified on the basis of its biochemical reactions using a conventional chromogenic substrate employed in RapID CB plus system Kit. The results are listed in table (4.2). As appeared from the data, *Arcanobacteriumpyogenes* was the most dominant genus of the isolated genera. This could explain the strong antimicrobial activity of soil that the bacterium isolated from. These results

are in agreement with that of others who confirmed the effective antimicrobial activity of other genera such as *Bacillus* particularly against the pathogenic microbes (Mohammed and Sheikh, 2010; Dhanapathi et al., 2008).

Table (4.2)
Biochemical reactions of Arcan.pyogenes sp. D12

Test	Experimental
Glu	+
Suc	+
Rib	+
Ma	+
AGlu	+
BGlu	_
NAG	_
Glyl	_
ONPG	_
PHs	+
EST	_
URT	_
PRO	_
TYR	_
PYR	_
LGLY	_
LEU	_
NIT	_

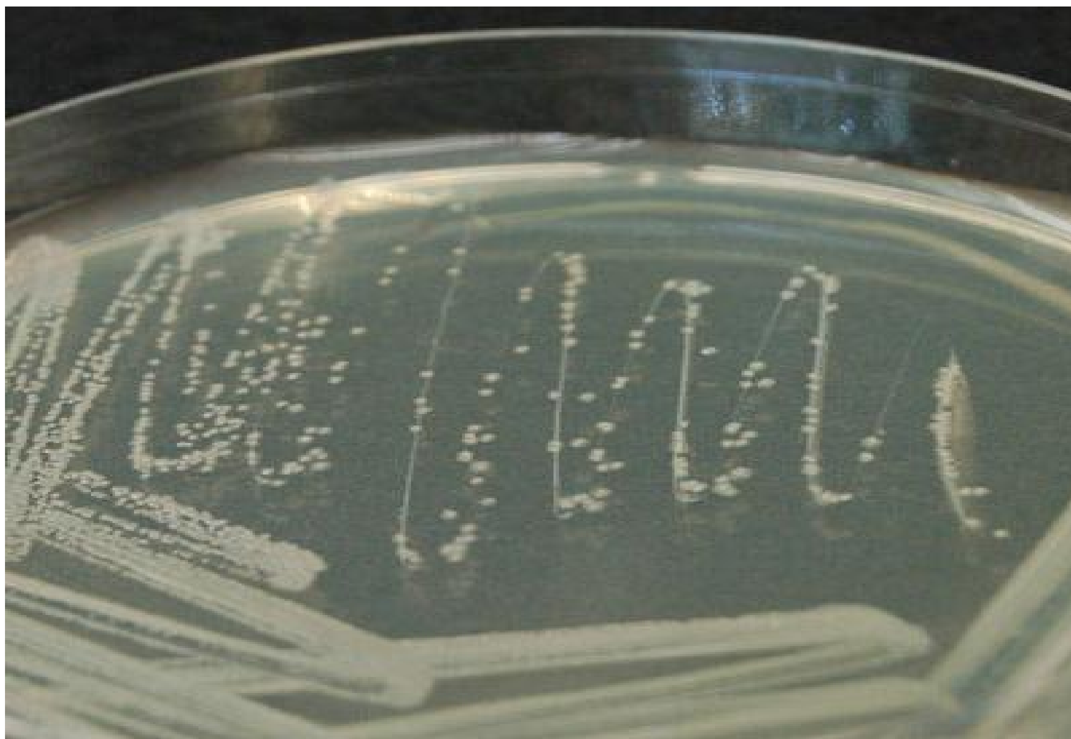


Figure (4)
Photograph of *Arcan.pyogenes*grown in NA medium

4.3 Small scale cultivation of *Arcan.pyogenes*sp. D12 and optimization of culture media for bioactive crude extract production :

*Arcanpyogenes*sp. D12 was cultivated in 1 L Erlenmeyer flasks containing 500 ml of LB media. It required four days to reach the stationary phase and the cultivation stopped after the decreasing in viable cell number indicating beginning of death phase after 7-8 days, with increase in pH ; to value pH 8.9 – 9.1 . The crude extract pH was adjusted to pH 8 and pH 4. the extract was active at the extracted pH values of 8. The amount of produced crude extract was 9 µg/ml. The resulting extract showed activity against the Gram positive bacteria *M. Luteus* , see Table (4.3). This is may be due to that the optimal condition of the growth of antibiotic producing microorganism is probably not favored.

Table (4.3)
Bioactivity of crude extract of *Arcanpyogenes*sp. D12 at different concentration

Test organism	Inhibition zone (mm)			
	PH 4		PH8	
	300 µg/disc	500 µg/disc	300 µg/disc	500 µg/disc
M. Luteus	*7*	8	9	11

As shown in the Table ; the crude extract is more effective at pH 8 .The inhibition zone was increased when increasing the concentration of extract. The resulting extract showed activity against *M.luteus*, whereas the gram negative bacteria were resistant to the metabolites of this strain. The same results were obtained by other researcher (Stauber and scherer , 1994 , Motta and Brandelli , 2004) . They showed that Bacteriocins were generally effective against gram positive bacteria (e.g. *Micrococcus* species, *Listeria* and *Anthrobacter*), while several tested gram negative bacteria were insensitive (e.g*Escherichia*and, *Enterobacter*). Also, Production of extended spectrum β-lactamase enzymes emerged in Gram negative bacteria and caused the infections to become more difficult to treat in view of their resistance to a wide range ofantibiotics (Abdel-Massih et al., 2010)

4.4 Large scale fermentation of *Arcan.pyogenes*sp. D12 :

*Arcanpyogenes*sp. D12 was cultivated in 1LX 20 flask of LB media at 27 ◦C and with 140 rpm agitation. The period incubation for cultivation process took between 7 – 8 days. Cultivation stopped once the strain entered the death phase. The pH value was increased to reach almost 9. Increasing in the pH could be interpreted as; cells started use the amino acids as nitrogen and carbon source. The antimicrobial activity started after 24 hours and reached its maximum value at the end of cultivation process . The filtrate was adjusted as previously mentioned to pH 8 and extracted with equal volume of ethyl acetate.

4.5 Preliminary purification of the secondary metabolites from the crud extract of Arcan.pyogenes sp. D12 :

Preliminary purification was done using Aluminium TLC plates impregnated with silica gel 60. The Rf values of developed bands are listed in table (4.6) and their migration profile is shown in figure (3). Our results show that all TLC fractions, exhibited more pronounced antibacterialactivity on *M.luteus* (Figure 5).

Table (4.4)
Migration rate of developed bands from crude extract of Arcan.pyogenes sp. D12

Band NO.	**1**	**2**	**3**	**4**
Rf	**0.02**	**0.23**	**0.4**	**1**

The bands 1,2,3 and 4 showed activity in bioautography is shown in Figure (3) . The compounds are semipolar as indicated from their Rf value.

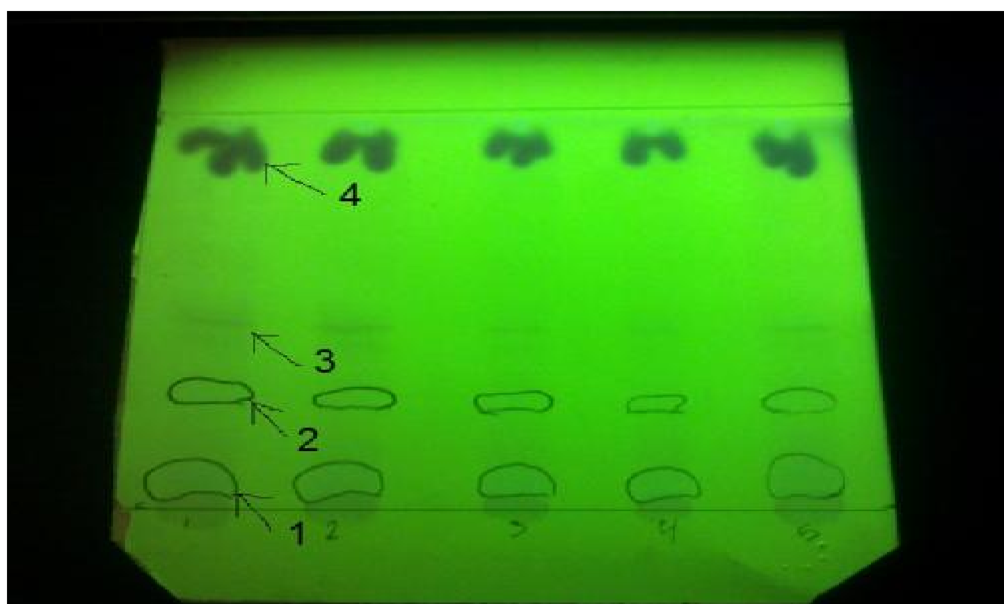


Figure (5)
Migration profile of the crude extract of Arcan.pyogenes sp. D12 on TLC (at 360 nm) .



Figure (6) :
Activity of bands of TLC on bioautography

As the incidence of multidrug resistant human pathogens is increased, looking for novel antibiotics has achieved new importance. Many clinically relevant microbes have developed resistances resulting from the exposure to sublethal concentrations of antibiotics in hospital environments but also in animal farms where antibiotics are used as growth enhancers (Witte, 1999). Finally, Changes in the growth throughout the incubation period may cause changes in physiology consequently affect antagonistic qualities of a particular strain. Within natural bacterial communities physiological changes are probably much more diverse and may result in much more variable antagonistic properties of individual strains than observed in the present study. Further studies on antagonistic interactions between different growths conditions should be focused more based on the case by case basis.

References

Atlas , R. M. And Bartha, R. (1998). **Fundamentals and applications. In: Microbial ecology**. 4th edition. Benjamin/Cummings Science publishing, New York.

Abdel-Massih, R., Elias Abdou, E., Baydoun, E., and Daoud, Z., (2010) **Antibacterial Activity of the Extracts Obtained fromRosmarinusofficinalis,** Origanummajorana, and *Trigonella*foenum-graecum on Highly Drug-Resistant GramNegative.

Bacilli . **Journal of Botany** Volume 2010, Article ID 464087, 8 pages doi:10.1155/2010/464087

AL-Saady , H.A.(2011).**Isolation of bioactive substances from soil-derived bacteria in Jordan (Tafeila area) and theirpotential as source of antimicrobial agents** . Master thesis, Mu'tah University.

AL-Tarawneh , A.H.(2011). **Isolation and molecular characterization of antibiotic producing bacteria from soil of (AL-Karak region) and their potential as source of antimicrobial agents** . Master thesis, Mu'tah University.

AL-Zereini , W.(2006). **Natural products from marine bacteria**. Ph.D thesis, Universität Kaiserslautern, Germany.

Bèrdy,J.(2005).**Bioactive microbial metabolites**.j.Antibiot.58(1):1-26

Bowman, J. P., McCammon, S. A., Brown, J. L., Nichols, P. D., andMcMeekin, T.A. (1997). Psychroserpensburtonensis gen. nov., sp.nov., and Gelidibactealgens gen. nov., sp. nov., psychrophilicbacteriaisolated from antarctic lacustrine and sea ice habitats. Int. J. Syst.

Bacteriol. 47(3): 670-677Ceylan, O., Okmen, G., and Ugur, A. (2008). **Isolation of soil Streptomyces assource antibiotics active against antibiotic- resistant bacteria**. EurAsianJournal of BioSciences. 73-82.

Clardy, J., Fischbach1, M., and Currie, C. (2009). **The history of antibiotics**. Curr Biol. 19(11): 437–441.

Clark, A.M.(1996). **Natural products as a resource for new drugs**.Pharmaceut. Res. 13/8: 1133- 1141.

Corstvet, R. E., Gentry, M. J., Newman, P. R., Rummage, J. A. and Confer, A.W. (1982). **Demonstration of age- dependent capsular material onPasteurellahaemolytica serotype I. Journal of clinical microbiology**.1123-1126.

Cragg, G. M., Newman, D.J. (2001). **Natural products drug discovery in the next millennium**. Pharmaceut. Biol., 39: 8-17.

Dhanapathi, Prabhakar, T.G., Prabhakar, P., 2008. **Antibacterial activity of *Bacillus subtilis* is extraction pathogenic organisms**. Tamil Nadu J. Vet. Anim. Sci. 4 (4), 150–153

Dancer, S.J. (2004). How antibiotics can make us sick: **the less obviousadverse effects of antimicrobial chemotherapy**. The Lancet InfectiousDiseases. 4: 61-611.

Dehnad, A., Parsa, L,. Bakhshi, R., AbdiSoofiani, S., and Mokhtarzadeh, A.(2010). **Investigation antibacterial activity of Streptomycetes isolates from soil samples**, West of Iran. African Journal of Microbiology Research Vol. 4(14): 1542-1549.

Demain, A.L. (1998). **Induction of microbial secondary metabolism. InternatlMicrobio**l. 1: 259–264.

Demain, A. L. (1998b). **Microbial natural products** : Alive and well in 1998.Nat. Biotechnol., 16(1): 3-4.

Derderian, S.L. (2007). Alexander fleming's miraculous discovery ofPenicillin. Rivier Academic Journal. Volume 3, number 2.El-Banna, N. (2006). **Effect of carbon source on the antimicrobial activity of Corynebacteriumkutscheri and Corynebacteriumxerosis**. African Journal of Biotechnology Vol. 5 (10): 833-835.

El-Banna , N., Samar, S. Quddoumi and Daradka, H. (2007). **Antimicrobial substances produced by bacteria isolated from different Jordanian sources that are active against methicillin-resistant Staphylococcus aureus.**

Falkinham, J.O. Wall, T. E., Tanner, J.R., Tawaha, K., Alali, F. Q., Li, C., and Oberlies, N.H. (2009). **Proliferation of Antibiotic-Producing Bacteria and Concomitant Antibiotic Production as the Basis for the Antibiotic Activity of Jordan's Red Soils**. Applied and Environmental Microbiology. 75: 2735–2741

Fenical, W. (1993). **Chemical studies of marine bacteria**: developing a new resource. Chem. Rev. 93(5): 1673-1683.

Fleming, A. (1929). **On the antibacterial action of cultures of Penicillium, with special reference to their use in the isolation of** B. influenzae. British Journal of Experimental Pathology. 10: 226-236.

Fleming, A. (1980). " **Classic in infectious diseases: on the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B**. influenzae by Alexander Fleming, Reprinted from the British Journal of Experimental Pathology 10:226-236.

Fitri,L. and Bustam, B. (2010). **Screening of antimicrobial producing strains isolated from the soil of grassland rhizosphere in**

PocutMeurahIntan Forest Park Seulawah, Aceh Besar. Biodiversitas.11: 129- 132.

Hentschel, U., Schmid, M., Wagner, M., Fieseler, L., Gernert, C., Hacker, J., 2006. **Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Med sponges *Aplysinaaerophoba* and *Aplysinacavernicola***. FEMS Microbiol. Ecol. 35 (3), 305–312.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., Williams, S.T., 1994. Bergey's**Manual of Determinative Bacteriology,** ninth ed. Williams and Wilkins, Baltimore, London.

Helmke, E., and Weyland, H. (1984). Rhodococcusmarinonascens sp. nov., an actinomycete from the sea. **International Journal of Systematic Bacteriology**. 34(2): 127-138.

Islam, Md.R., Jeong, Y.T., Ryu, Y.J., Song, C.H. and Lee, Y.S. (2009).**Isolation, identification and optimal culture conditions of Streptomyces albidoflavus C247 producing antifungal agents against Rhizoctoniasolani AG2-2. Mycobiology**. 37(2) : 114-120.

Lay, W. B. (1994) **Microbes analysis in laboratory**. Raja GrafindoPersada Jakarta. [Indonesia]

Motta, AS., Cladera-Olivera, F. and Brandelli, A. (2004). **Screening for antimicrobial activity among bacteria isolated from the Amazon basin. Brazilian Journal of Microbiology**. 35:307-310.

Mohammed, H., Sheikh,A. (2010). **Antimicrobial activity of certain bacteria and fungi isolated from soil mixed with human saliva against pathogenic microbes causing dermatological diseases**. **Saudi Journal of Biological Sciences**. 17 (4) 331–339

Miyadoh, S. (1993). **Research on antibiotic screening in Japan over the last decade: Aproducing microorganisms approach**. Actinomycetologica. 9:100-106.

Nakanishi, K. (1999). **An historical perspective of natural products chemistry**. Comprehensive Nat. Prod.Chem., 8, xxi-xxxviii.

Nanjwade, B.K., Chandrashekhara, S., Shamarez,A.M., Goudanavar, P.S. and Manvi, F.V. (2010). **Isolation and morphological characterization of antibiotic producing Actinomycetes**. Tropical Journal of Pharmaceutical Research June. 9 (3): 231-236

.

Narendra., K., Singh., R.K., Mishra, S.K., Singh, A.K., and Pachouri, U.C. (2010). **Isolation and screening of soil Actinomycetes as source of antibiotics active against bacteria. International Journal of Microbiology Research**. Volume 2 :. 12-16.

Nelson, M.L. (1998).**Chemical and Biological Dynamics of Tetracyclines**. Advances in Dental Research. 12:5-11.

O,Donnell, A. G., Norris, J. R., Berkeley, R. C. W., Claus, D., Logan, K. A.and Nozaki, R. (1980). **Characterization of Bacillus subtilis, Bacillus pumilus, Bacillus licheniformis, and Bacillus amyloliquefaciens by Pyrolysis Gas-Liquid Chromatography, Deoxyribonucleic Acid- Deoxyribonucleic Acid Hybridization, Biochemical tests, and API systems**. InternationajloUrnal of SystematbiaccTeriologaYp. Vol.30, No. 2. 448- 459.

O,Keefe, B. R. (2001). **Biologically active proteins from natural product extracts**. J. Nat. Prod., 64, 1374-1381.Petrossian,G.(2012). Discovery and Isolation of Bioactive Natural Products from Actinomycete Bacteria

petrossia,G (2012). **Discovery and Isolation of Bioactive Natural Products from Actinomycete Bacteria With an Examination of Infectious Disease Drug Development and Application**: A Comparison of the Developing and Developed Worlds

Priest, F. G., Goodfellow, M., Shute, L. A. and Berkeley, R. C. W. (1987).**Bacillus amyloliquefaciens sp**. Nov. norn. Rev. InternationajloUrnalofSystematbiaccTeriologjaYn . Vol. 37. No. 1, 69-71.

Saadoun, I., Wahiby, L., Ababneh, Q., Jaradt, Z., Massadeh, M. and ALMomani, F. (2008). **Recovery of soil streptomycetes from arid habitats in Jordan and theirpotential to inhibit muli-drug resistant Pseudomonas aeruginosa pathogens**. World. J. Microbiol. Biotechnol. 24:157-162

Saha, M.R., Ripa, F.A., Islam, M.Z. and Khondkar, P. (2010). **Optimization of Conditions and in Vitro Antibacterial Activity of Secondary Metabolite Isolated from Streptomyces sp**. MNK7. Journal of Applied Sciences Research. 6(5): 453-459.

Steel, K. J.(1961). **The oxidase reaction as taxonomic tool. Journal of Stone**, N. E., Fan, J. B., Willour, V., Pennacchio, L. A., Warrington, J. A.,strategies for biotechnology: the paradigm shift. Microbiol. Mol. Biol.

Rev. Strohl, W. R. 2000. **The role of natural products in a modern drugdiscovery program**. Drug Discovery Today. 5: 39-41.

Süßmuth, R., Eberspächer, J., Haag, R., and Springer, W. (1999).**MikrobiologischbiochemischesPraktikum. Georg ThiemeVerlag,Stuttgart**.Technical Manual, pGEM®-T and pGEM®-T Easy Vector Systems, printed in USA revised 12/10.

Witte. W. (1999)**Antibiotic resistance in Gram-positive bacteria**: epidemiological aspects, J. Antimicrob. Chemother., 44, Suppl. A1–9.

Wulff, E.G., Mguni, C.M., Mansfeld-Giese, K., Fels, J., Lübecka, M. andHockenhull, J.(2002). **Biochemical and molecular characterization of Bacillus amyloliquefaciens, B. subtilis and B**. pumilus isolates with distinct antagonistic potential against Xanthomonascampestrispv. Campestris. Plant Pathology. 51: 574–584.

www.wiley.com/college/pratt/0471393878/student/activities/bacterial_drug_re sistance/index.html

Ying, Z. (2007). **Mechanisms of Antibiotic Resistance in the Microbial World**.ClinPharmacolTher. 82: 595-600.

المعلومات الشخصية

الاسم: ميساء محمود الحوراني

التخصص: ماجستير العلوم الحياتية

الكلية: العلوم

السنة: 2014

هاتف رقم: 0797074283

032341924

البريد الإلكتروني: maisa.horani@hotmail.com